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Chemistry

Identification of highest-affinity ligands by affinity selection from equimolar peptide mixtures generated by robotic synthesis

(chemical diversity/peptide library/multiple-peptide synthesis)

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ABSTRACT **A fully automated peptide synthesizer has been constructed that is capable of the simultaneous synthesis of up to 36 individual peptides and the synthesis of equimolar peptide mixtures. The instrument consists of an array of reaction vessels, a series of solenoid valves to control liquid flow, and a Zymark robot to deliver solvents and reagents; all components are computer controlled and coordinated. Equimolar peptide mixtures are obtained by algorithms that automate the mixing and distribution of peptide–resin particles. This technology was used to synthesize a library of 361 peptides, generated by randomizing two critical binding residues of a 10-mer epitope known to bind an anti-human immunodeficiency virus gp120 monoclonal antibody. Each critical residue was substituted with 19 amino acids consisting of all the natural amino acids except cysteine. The library was synthesized as 19 pools, each containing 19 peptides. Each pool was screened in a solution-phase competition ELISA assay. The 12 most inhibitory peptides in the library were isolated by a rapid affinity-selection method and were identified by mass spectrometry and amino acid analysis. The binding properties of these 12 selected peptides were verified by synthesis and assay of the individual peptides. The two critical residues investigated were found to contribute independently to antibody binding.**

The chemical diversity and step-wise synthesis of short peptides have made them attractive candidates for the rapid generation of biologically active lead compounds in drug-discovery programs. In recent years chemists and biologists have devised multiple- and mixed-peptide approaches to achieve sequence diversity (1). Multiple-peptide synthesis technology allows a substantial increase in the number of individual peptides that can be generated and screened, as compared with conventional synthesizer technology. The Geysen pin method (2), the Houghten tea-bag method (3), and the use of cellulose discs (4) allow parallel synthesis of several hundred peptides in a period of weeks. Recently, automated instruments have been designed that are capable of synthesizing up to 96 individual peptides simultaneously (5, 6). Light-directed spatially addressable parallel chemical synthesis (7), a method requiring the use of photolithographic equipment, allows simultaneous synthesis of $\approx 10^4$ peptides on a solid phase. Resin-bound synthetic peptide libraries have been generated that contain $\approx 10^6$ ligands (8). Finally, epitope libraries, where peptide sequences are displayed on the surface of filamentous bacteriophage particles (9–11), are a source of great peptide diversity (10^7–10^8 distinct sequences). Although the latter three methods generate a large number of compounds, the C termini of these peptides are anchored to a surface, thus preventing assay of the free peptide in solution. The qualitative screening of free-peptide libraries that contain $\approx 10^6$ ligands has recently been reported (28).

We describe here a fully automated procedure using standard 9-fluorenylmethoxycarbonyl (FMOC) chemistry and polystyrene resin, that allows the synthesis of solution-phase (or resin-bound) peptide libraries. The components of these libraries are synthesized in equimolar proportions by physically separating the solid support (resin) into *n* equal aliquots, coupling a unique amino acid to each aliquot, and then mixing all of the resin aliquots (12) (Fig. 1). After removal from the resin, the peptide mixtures are characterized by amino acid analysis and mass spectrometry to verify their composition. The mixtures are then screened in a competition binding assay to ascertain whether they contain high-affinity components.

An affinity selection method is also described in which an antibody was used to select the highest affinity peptides from an equimolar mixture of peptide analogs. The selection process was performed with both the ligand mixture and the receptor in the solution phase, under conditions where the ligand components compete for a limited number of receptor binding sites. This allows a quantitative interpretation of the receptor-binding properties of the mixture components with the highest affinity.

MATERIALS AND METHODS

FMOC-protected amino acids and polystyrene resins were obtained from Advanced ChemTech. Side chain protecting groups were *tert*-butyl esters for aspartic and glutamic acids; *tert*-butyl ethers for serine, threonine, and tyrosine; triphenylmethyl for cysteine, histidine, asparagine, and glutamine; *tert*-butoxycarbonyl for lysine; and 2,2,5,7,8-pentamethylchroman-6-sulfonyl for arginine. 4-(α-[2′,4′-Dimethoxyphenyl]-FMOC-aminomethyl)phenoxy resin (100–200 mesh, 1% crosslinked with divinylbenzene) was used at a 0.40 mmol/g substitution level for the synthesis of C-terminal amides (13). Reagent grade dimethylformamide (Baxter Scientific Products), diethyl ether (Mallinckrodt), piperidine (Aldrich), and 1,2-dichloroethane (Aldrich) were used without further purification.

HPLC characterization of the peptides was performed on a Rainin HPX system controller with a C_{18} reversed-phase HPLC column (Vydac, 25 cm × 4.6 mm) and a gradient elution with eluants A [200 mM triethylammonium phosphate (pH 2.5)] and B [60% (vol/vol) acetonitrile in A], giving 10–40% B in 35 min.

Amino acid compositions were obtained by using the Pico-Tag method of Waters (14). Mass spectra were analyzed in a glycerol matrix by liquid-matrix secondary-ion mass

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Abbreviations: FMOC, 9-fluorenylmethoxycarbonyl; EPM, equimolar peptide mixture; mAb, monoclonal antibody.

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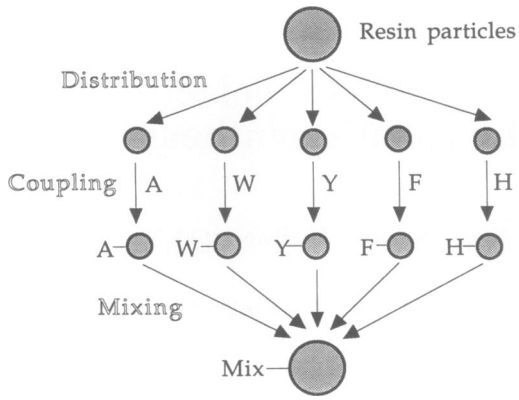

FIG. 1. Equimolar peptide mixtures (EPMs) are synthesized by splitting a peptide-resin into equal portions, coupling a unique amino acid to each portion, and then recombining the resin samples. In this example, an equimolar mixture of alanine-, tryptophan-, tyrosine-, phenylalanine-, and histidine-coupled resin is obtained. This procedure can be repeated to generate mixtures of higher complexity.

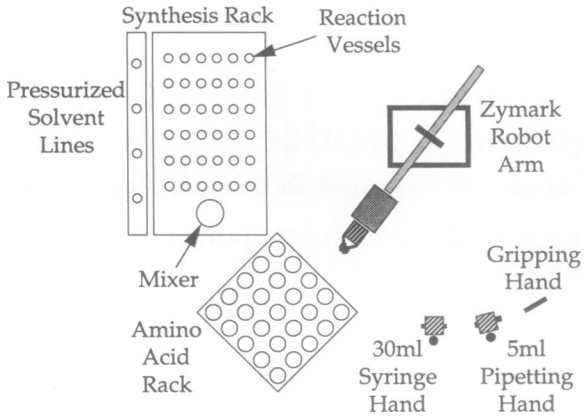

FIG. 2. The EPM synthesizer consists of an array of reaction vessels, a rack to hold amino acid solutions, a resin-mixing chamber, pressurized solvent lines, and three interchangeable hands, all of which are accessed by a central Zymark robotic arm.

spectrometry on a VG analytical ZAB 2SE mass spectrometer at Mass Search (Modesto, CA).

Monoclonal Antibody (mAb). Mice were hyperimmunized (15) with a recombinant form of glycoprotein gp120 from the SF2 isolate of human immunodeficiency virus type 1 (HIV-1) produced in genetically engineered mammalian cells (16). The IgG was purified from ascites fluid by affinity chromatography on protein G-Sepharose (Pharmacia).

Peptide Synthesis. The standard Merrifield solid-phase technique was used with diisopropylcarbodiimide/1-hydroxybenzotriazole *in situ* activation chemistry (17). FMOC-protected amino acids were added to the peptide-conjugated resin in 5-fold molar excess to amino groups at a concentration of 0.3 M. Coupling times were 30 min, and each position was doubly coupled. Unreacted amino groups were capped with acetic anhydride after the coupling cycle. The FMOC group was deprotected with 20% piperidine/dimethylformamide (one treatment for 2 min followed by one for 20 min). Total cycle time for 19 peptides was ≈3 hr per residue.

Deprotection of the peptide side chains and cleavage from the resin were accomplished by treatment with 82.5% trifluoroacetic acid/5% phenol/5% water/5% thioanisole/2.5% ethanedithiol (10 ml per 500-mg sample of resin) (18). The deprotection mixture was incubated at room temperature for 2 hr, and concentrated to a 1-ml volume with gentle heating under a stream of nitrogen. The peptide residue was then diluted with 10 ml of aqueous 10% (vol/vol) HOAc/H_2O and extracted three times with 15 ml of diethyl ether. The peptides were lyophilized and used without further purification.

Robotics. The EPM synthesizer was designed around a Zymate XP laboratory automation system (Zymark, Hopkinton, MA), which consists of a central robotic arm that can exchange hands, thereby providing a variety of functions. The synthesizer also consists of an array of fritted reaction vessels, a resin mixing vessel, a series of solenoid valves, a rack to store amino acid solutions, pressurized solvent bottles, a syringe pump, a vacuum trap, and a computer controller (Fig. 2).

The user inputs the peptide sequence information, adds resin to the appropriate reaction vessels, prepares the amino acid and activator solutions, and fills the solvent reservoirs. The robot then adds the appropriate amino acid solutions (stored under a blanket of argon) to the desired reaction vessels with a pipetting hand. The hand changes pipet tips when necessary to avoid cross-contamination. Small volumes of activating reagents are then added via a gripping hand, which holds a reagent line connected to a syringe pump. Thorough mixing of the reaction mixture is achieved by the bubbling argon gas through the fritted bottom of each reaction vessel. The solvents or reagents are removed by the application of a vacuum, which draws the liquid through the frit. Fresh wash solvent is added by the gripping hand, which dispenses the solvent from a pressurized reagent line. Liquid flow is regulated by Teflon solenoid valves (General Valve, Fairfield, NJ) that are controlled from a digital I/O board (GW Instruments, Somerville, MA) inside a Macintosh II computer (Apple Computer, Cupertino, CA). The entire plumbing and solvent path is constructed from Teflon, siliconized glass, polyethylene, or polypropylene. Details of the synthesizer construction are described elsewhere (19, 20). The Macintosh software for control of the solenoid valves and for interfacing with the Zymark controller was written in Microsoft QuickBasic and Think C languages.

The EPM synthesizer uses a custom hand, fitted with a 30-ml syringe to perform the task of resin separation and mixing. Distribution of peptide–resin samples into equal portions is performed by dispensing equal volumes of a 3% (wt/vol) resin slurry in 60% 1,2-dichloroethane in dimethylformamide. The resin is thoroughly mixed (by argon bubbling) just prior to distribution to ensure a uniform resin slurry. The density of this solvent system allows for a uniform suspension of the resin and an equimolar distribution of resin by volume, with minimal interruption of the synthesizer chemistry (21).

Affinity Selection. The anti-gp120 mAb (1.0 nmol, IgG, Chiron 26-8-F8-E3) was incubated with a 19-component mixture (Arg-Ala-Xaa^1-His-Thr-Thr-Gly-Arg-Ile-Xaa^2, where Xaa^1 is a fixed amino acid and Xaa^2 is a mixture of 19 amino acids; this is referred to in single letter code: $RAX^1HTTGRIX^2$) at a 10-fold molar excess of each peptide (10 nmol) in 200 mM NaCl/10 mM phosphate buffer, pH 7.5 (100 μl) for 1 hr at room temperature. The mixture was then fractionated by gel filtration on a Sephadex G-25 fast desalting column (Pharmacia, 1 × 10 cm) in 150 mM NaCl/10 mM phosphate buffer, pH 7.5 at a flow rate of 3.0 ml/min. The first peak collected (the breakthrough fraction) was the antibody–peptide complex. The peptides were then dissociated from the antibody in 1% aqueous trifluoroacetic acid and analyzed by reversed-phase HPLC. The selected peptide peaks were collected and characterized by mass spectrometry and amino acid analysis.

ELISA. Peptide pools were assayed in a competition ELISA format over a concentration range of 0.1 nM–500 μM for each peptide. Immulon 1 microtiter plates (Dynatech) were coated overnight at 4°C with a recombinant gp120 antigen (env 2-3; 0.2 μg per well in 50 mM borate, pH 9.0).

A 50-μl aliquot of the peptide pool was incubated with 50 μl of diluted IgG (26 μM stock diluted 1:50,000) in 0.5 M NaCl/1% Triton X-100/0.1% casein/250 mM phosphate buffer, pH 7.5 for 1 hr at 37°C. The plates were washed six times with wash buffer (150 mM NaCl/0.5% Triton X-100) and incubated with 100 μl of horseradish peroxidase-conjugated goat anti-mouse antibody (stock solution of 1 mg/ml diluted 1:1000; Boehringer Mannheim) for 1 hr at 37°C. The plates were washed as above, and the bound conjugated antibody was quantified by color development with 100 μl of *o*-phenylenediamine at 5 mg/ml in 50 mM sodium citrate/0.02% H_2O_2, pH 5.1. Plates were read at 450 nm on a Molecular Devices (Menlo Park, CA) ThermoMax microplate reader.

RESULTS

EPM Synthesis. To synthesize EPMs by the resin-splitting algorithm, it is first necessary to demonstrate (*i*) the synthesis of individual peptides in high purity and (*ii*) the precise separation of peptide–resin into aliquots of equal size. The purity of peptides synthesized on the EPM synthesizer was demonstrated by the simultaneous synthesis of 12 individual $RAX^1HTTGRIX^2$ analogs (where X^1 was fixed as phenylalanine, tryptophan, tyrosine, or histidine and X^2 was varied among proline, isoleucine, and leucine). Each peptide was synthesized in >95% purity as determined by analytical HPLC (three of these are shown in Fig. 3). The identity of these 12 peptides was confirmed by mass spectrometry and amino acid analysis (data not shown).

The precision with which the EPM synthesizer distributes equal portions of resin was determined during the synthesis of the $RAX^1HTTGRIX^2$ pools. The first step involved the partition of 0.95 mmol of unsubstituted resin into 19 equal portions. After partitioning, the dry weights of each of these aliquots were determined to be within a standard deviation of ±3% from the mean. A different amino acid was then coupled to each of the 19 resin aliquots, after which the resin portions were combined. Amino acid analysis of this resin sample (Fig. 4*a*) indicated that the mixture is equimolar to within a standard deviation of ±14%. The remainder of the $RAX^1HTTGRIX^2$ pools was synthesized by coupling additional amino acids to this resin mixture. When the X^1 position was reached, the resin particles were again split into 19 aliquots, and the last three amino acids were coupled in separate tubes. The resulting 19 pools were deprotected and cleaved from the resin as separate samples. The 19-component soluble mixture where X^1 was tryptophan was analyzed by amino acid analysis (Fig. 4*b*) and mass spectrometry (data not shown), showing that all components were present in approximately equimolar proportions.

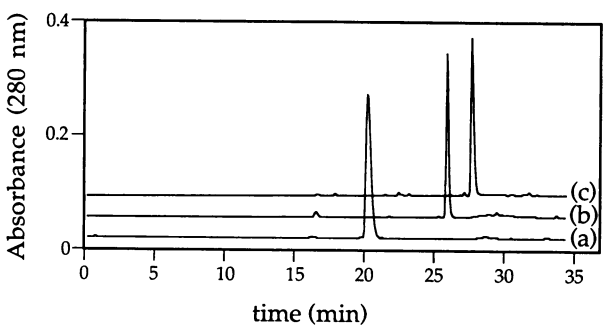

FIG. 3. Analytical reversed-phase HPLC chromatograms of three crude peptides individually synthesized on the EPM synthesizer. Peaks: a, Ac-RAWHTTGRIP-NH_2; b, Ac-RAWHTTGRII-NH_2; and c, Ac-RAWHTTGRIL-NH_2. These peptides were synthesized to confirm the identity of the affinity-selected peptides.

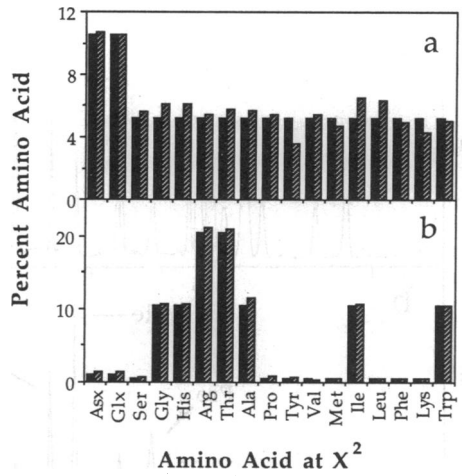

FIG. 4. Amino acid analysis was used at two stages of the mixture synthesis to determine the equimolarity of the $RAWHTTGRIX^2$ pool. (*a*) A resin sample of the 19-component peptide mixture with a variable C-terminal amino acid. (*b*) The cleaved 19-component mixture $RAWHTTGRIX^2$. ■, Predicted values; ▨, observed values.

Identification of Antibody-Binding Peptide Analogs. An anti-gp120 mAb was used as a model for peptide–receptor interactions. This mAb was elicited to a yeast-derived recombinant form of the gp120 protein (env 2-3) and was mapped to a 10-mer sequence (Arg-Ala-Phe-His-Thr-Thr-Gly-Arg-Ile-Ile) within the V3 loop using gp120 deletions (22). The critical residues of this epitope were determined to be Phe-3, Arg-8, and Ile-10 by the synthesis and assay of the alanine-substituted analogs at each position of the 10-mer epitope (data not shown).

The mixture technology was then used to investigate the pairwise interactions of two critical residues (Phe-3 and Ile-10) by substituting each position with all of the other natural amino acids except cysteine: Ac-$RAX^1HTTGRIX^2$-NH_2, where both the fixed X^1 and the variable X^2 positions contain 19 amino acids. This generated a library of 361 peptides. The library was synthesized as 19 subpools, each containing 19 peptides. Specifically, each pool contained a unique (fixed) amino acid at X^1 and a mixture of 19 amino acids at X^2. The pools were assayed over a wide range of concentrations (0.1 nM–500 μM) in an ELISA assay measuring competition (in solution) for free IgG with immobilized gp120 (Fig. 5). The IC_{50} values reported for the 19 pools represent the molar concentration of each peptide. The four most inhibitory pools contained the aromatic amino acids phenylalanine, tryptophan, tyrosine, and histidine at X^1.

To identify the amino acids in the X^2 position, each of the four most inhibitory pools was affinity-selected with the mAb. Specifically, 10 μM IgG was incubated with a 19-

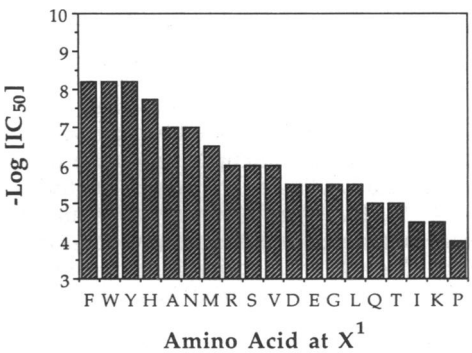

FIG. 5. Competitive inhibition of the mAb–gp120 interaction by the 19 $RAX^1HTTGRIX^2$ peptide pools. Each pool has a different but fixed residue at X^1 and a mixture of 19 amino acids at X^2.

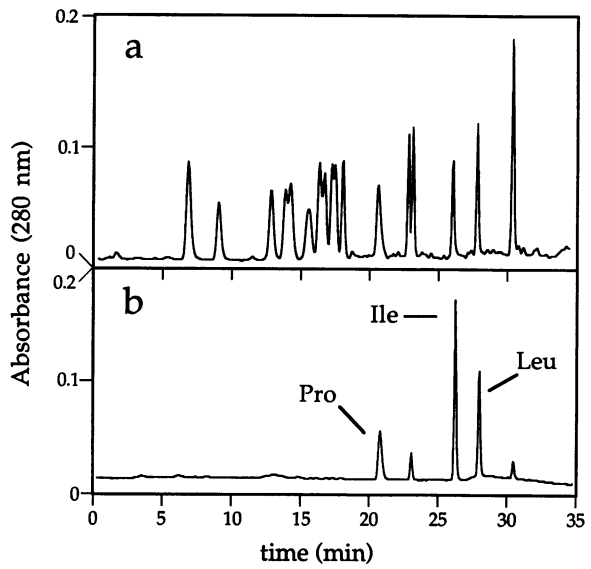

FIG. 6. Affinity-selection experiment with the RAWHTTGRIX^2 pool, where X^2 varies among 19 amino acids. Analytical reversed-phase HPLC analysis of the 19-component peptide mixture (*a*) and the same mixture after affinity selection with an anti-gp120 mAb (*b*).

component mixture at a 10-fold excess of each peptide for 1 hr at room temperature. The IgG–peptide complex was separated from the excess free peptides in <1 min by gel-filtration chromatography. The peptides that were bound were further analyzed by reversed-phase HPLC (Fig. 6). All four pools showed three major peaks with similar relative peak areas. These components were identified by mass spectrometry, amino acid analysis and HPLC retention time (Fig. 3). In all four cases, X^2 was proline, leucine, and isoleucine. The affinities of the selected peptides were confirmed as individual peptides (Table 1).

DISCUSSION

EPM Synthesis. A robotic EPM synthesizer was constructed (19, 20) that allows the synthesis of solution-phase peptide libraries. Standard FMOC-amino acids and polystyrene resins are used with carbodiimide, benzotriazol-1-yl-oxy-tris(pyrrolidino)phosphonium hexafluorophosphate (PyBOP), or 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) activation chemistries. Although the EPM synthesizer can be operated in a multiple-peptide synthesis mode similar to commercially available synthesizers (5, 6), the synthesizer has the additional capability to quantitatively transfer peptide–resin particles in and out of reaction vessels. This allows the synthesis of peptide libraries of known composition. Specifically, EPMs are synthesized by physically splitting the solid support (resin) into *n* equal aliquots, coupling a unique amino acid to each aliquot, and then combining all of the resin aliquots (12) (Fig. 1). In an alternative method, a mixture of activated amino acids is coupled to a single batch of resin (23). This amino acid mixture method can be used to synthesize equimolar peptide mixtures only if the concentration of each amino acid is adjusted to compensate for its coupling rate (ref. 24; V. Huebner, personal communication). The former resin-splitting method has the advantage that mixtures will be much closer to their designed composition, since the coupling reactions are performed in separate vessels and driven to completion; thus, no knowledge of individual rate constants is required. Theoretically, the number of beads ($\approx 10^6$ per ml) limits the library complexity when conventional peptide synthesis resins are used. This is because the resin-splitting algorithm generates one peptide sequence per bead (8). Therefore, to ensure a complete library and equimolarity of the mixture components, the library complexity must be considerably less than the number of beads.

Table 1. Identification and binding affinities of the affinity-selected peptides

X^1	Peak	AA at X^{2*}	$-\log[IC_{50}]$	Relative peak area, % Predicted†	Relative peak area, % Observed
F	1	P	7.5	14	17
	2	I	8.1	58	45
	3	L	7.8	28	38
W	1	P	7.6	17	27
	2	I	8.1	56	42
	3	L	7.8	27	31
Y	1	P	7.5	14	26
	2	I	8.1	58	44
	3	L	7.8	28	31
H	1	P	7.3	29	29
	2	I	7.4	36	40
	3	L	7.4	36	31

*This was determined by mass spectrometry and amino acid (AA) analysis.
†These were calculated from the molar IC_{50} values of the individually synthesized peptides.

The EPM synthesizer allows the synthesis of libraries containing mixture residues and constant residues. A generic library intended for the random screening of a variety of receptors or enzymes, for example, can be synthesized in which a peptide contains a mixture of amino acids at every position. Alternatively, a specific library designed to probe structure activity relationships of peptide analogs can be synthesized in which a mixture is introduced at specific positions of the peptide (25). The amino-terminal residues can be held constant, generating a family of related mixtures.

To determine the equimolarity of the peptide mixtures synthesized on the EPM synthesizer, analyses were performed at various stages of the synthesis. The initial partitioning of the resin into 19 aliquots (prior to coupling of the C-terminal residue) occurred to within ±3% of the mean as determined by weight. After coupling the first residue to each aliquot and mixing the resins, amino acid analysis (Fig. 4*a*) indicated that the mixture was approximately equimolar. Mass spectrometry of the final X^1 = Trp soluble pool indicates that each peptide was in fact present, and amino acid analysis (Fig. 4*b*) indicates that the concentration of each peptide was within ±20% of the expected value (the accuracy of amino acid analysis is within ±10%). This confirms the approximate equimolarity of the mixture, and suggests that the amino acid-coupling yields and resin-mixing steps were adequate.

Screening and Affinity Selection of EPMs. Rapid identification of the subset of highest affinity receptor-binding peptides within a peptide library theoretically can be accomplished in two ways: (*i*) by the resynthesis and assay of each individual peptide (or as smaller subpools) (26, 28) or (*ii*) by the fractionation of the mixture by affinity selection, followed by analysis of the receptor-binding peptides. The synthesis of a mixture of peptide analogs that differ only in the identity of a particular residue(s) allows the comparison of the contribution of this residue(s) to the binding affinity (27). The screening of a family of 19 peptide mixtures in a competition-binding assay was used here to identify the most inhibitory pools as candidates for further analysis (Fig. 5). The affinity selection method was then used to identify receptor-binding components directly, thereby greatly reducing the number of peptides to be resynthesized.

A fundamental advantage of the affinity selection method is that it allows a pool of peptides to compete for binding to a limiting amount of receptor in solution. This is followed by

the rapid separation of the peptide–receptor complex from free peptides by gel-filtration chromatography. The peptides can be recovered from the receptor by methods that are not destructive to the receptor. When used with equimolar ligand mixtures, the affinity selection method has the additional advantage of allowing a quantitative interpretation of the relative binding affinities of the most inhibitory components. Under conditions where each peptide component of the mixture is in excess of the receptor, the composition of the receptor-bound peptides at equilibrium is directly proportional to their association constants. Thus, the major products obtained are the tightest-binding peptides.

The conditions of the affinity selection experiments were determined by the amount of peptide necessary for analysis and by consideration of the expected antibody affinities. Approximately 0.1 nmol of a peptide is necessary for mass spectrometry and amino acid analysis; therefore, an ample quantity (1.0 nmol) of the antibody was used. The antibody was used at a concentration (10 μM) that exceeds the K_d of the desired binders to ensure the selection of high-affinity peptides. Only peptides with K_d values substantially lower than the antibody concentration will be substantially bound. The ligand affinities that can be detected under these conditions were empirically determined to be <1–10 μM (data not shown). Mixture complexity is limited by peptide solubility. Accommodation of solubility by dilution of the selection mixture ultimately results in lowering the receptor concentration below desired levels.

The apparent association constant of an equimolar ligand mixture (K_{app}) of complexity n is a measure of the sum of affinities of the individual components (K_i):

$$K_{app} = \sum_{i=1}^{n} K_i.$$

Therefore, the individual component with the highest affinity in such a mixture will have an affinity between K_{app}/n and K_{app} (assuming there are no significant peptide–peptide interactions). The higher the complexity of the mixture, the larger the range of possible affinities. In the present study, the three most inhibitory RAX^{1}HTTGRIX^2 pools (where X^1 = phenylalanine, tryptophan, or tyrosine) were 19-component mixtures that exhibited an apparent IC_{50} of 6.3 nM. This could consist of one peptide with a 6.3 nM IC_{50} and 18 nonbinding peptides, or at the other extreme, 19 peptides—each with a 120 nM IC_{50}. The affinity selection method effectively distinguished between these two extremes, showing that there were three major peptide components that bound with significantly greater affinity than the rest of the mixture components (Fig. 6).

Of the 361 peptides tested by this method, only two other peptides [RAWHTTGRII and RAYHTTGRII with tryptophan and tyrosine as X^1 and isoleucine as X^2] were found to have an affinity as high as the parent peptide. All 12 of the most inhibitory peptides identified from the affinity-selection experiments had affinities within 1 order of magnitude to the parent peptide RAFHTTGRII with phenylalanine at X^1 ($-\log[IC_{50}] = 8.2$) and at least 10-fold higher than negative control peptides RAAHTTGRII with alanine at X^1 ($-\log[IC_{50}] = 6.4$) and RAFHTTGRIA with alanine at X^2 ($-\log[IC_{50}] = 6.4$). The relative affinities of the three selected peptides in each of the four pools examined above are proportional to the peak areas observed after affinity selection (Table 1), a condition that only holds when the mixture has equimolar components. From the relative peak areas for the X^1 = Trp pool (Fig. 6*b*), the predicted relative affinities are peptides with Ile > Leu > Pro in the X^2 position. This trend was confirmed by the IC_{50} determination of the individual peptides (Table 1). Furthermore, since there were a total of 19 peptides present in the original pool and only five peaks detected by selection, it can be concluded that the other 14 peptides in this pool must have affinities that are <1% of the peptide with isoleucine as X^2. The maximum possible affinity for any individual peptide in the other 15 less inhibitory pools is $-\log[IC_{50}] \leq 7.0$ (Fig. 5). The fact that the same three amino acids, proline, isoleucine, and leucine, appear at the X^2 position for the four best X^1 pools indicates that these two hydrophobic positions contribute independently to antibody binding.

A fully automated instrument has been developed that synthesizes equimolar peptide mixtures. These mixtures can be assayed in solution-phase competitive assays, allowing the affinity of the solution conformation of these peptides to be evaluated. Screening and affinity-selection methods can be extended to use mixtures of higher complexity and mixtures that contain nonstandard amino acids or conformational constraints. Mixture screening, when used in conjunction with equimolar peptide synthesis and an affinity-selection method, represents a powerful technology for the rapid identification of the highest affinity ligands in a peptide library.

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